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SYNTHESIS AND FORMULATIONS OF LIPID AMINOGLYCOSIDE CONJUGATES: NANOPARTICLES FOR EFFICIENT GENE AND siRNA DELIVERY

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ABSTRACT

Objective: To design and evaluate efficient lipid-aminoglycoside conjugates for the delivery of genes for gene therapy, and also for the delivery of siRNA molecules to knock down gene expression in mammalian cells

Methods: 3 β -[5''-(aminoethanethiol)-neomycin B] carbamoyl cholesterol (NeoChol) was synthesized. The abilities of this novel compound to condense DNA and to form nanoparticles were studied using ethidium bromide fluorescence quenching and nanoparticle characterization techniques. Transfection efficiency was studied in FEK4 primary skin cells and in an immortalized cancer cell line (HtTA), and compared with the non-liposomal transfection formulation Lipogen, *N*⁴,*N*⁹-dioleoyl-1,12-diamino-4,9-diazadodecane. Also, the abilities of this compound to condense siRNA and to form nanoparticles were studied using a Ribo Green intercalation assay and its abilities to deliver siRNA into cells were studied in FEK4 and HtTA cells using fluorescein-labelled *Label IT*[®] RNAi Delivery Control, a sequenced 21-mer from Mirus.

Results: We show efficient pEGFP and siRNA formulation and delivery to primary skin (FEK4) and cancer cell lines (HtTA).

Conclusion: Synthetic cationic lipid, a conjugate derived from neomycin B antibiotic and cholesterol is highly efficient for in vitro delivery of DNA and fluorescent siRNA.

Keywords: ??

INTRODUCTION

Nucleic acids show great promise as new therapeutics to treat both acquired and inherited diseases. One of the greatest challenges to the successful application of poly-nucleic acid drugs (DNA or siRNA) is the development of an efficacious delivery method [1]. Delivery systems are needed to compact genetic material into nanostructures that can be uptake by cells [2], to protect (poly-)nucleic acids from enzymatic damage during cellular transport, and to provide the possibility of targeting the delivery to specific cell types [3,4].

Viral vectors are still the most effective and commonly used method of DNA transport even though many problems with this delivery method have been revealed, e.g. immunogenicity, toxicity, safety issues, and problems in industrial quality control and scaling-up.[5] Polymeric cationic vectors e.g. polyethylenimine (PEI), exhibit efficient gene delivery, but are also cytotoxic [6]. Conversely, chitosan, a polymer of glucosamine, is non-toxic yet it displays low gene delivery efficiency in many cell lines [7].

Lipopolyamine-mediated gene delivery has emerged as a viable alternative to viral-based and polymer-based transfection systems [8,9], as polyamines, e.g. spermine and spermidine, are naturally occurring (catabolised within cells) and they have DNA-binding affinity, whilst inducing neither immune nor inflammatory responses. The synthetic gene vectors based on these polyamines [10,11] normally incorporate 3 parts: polyamine scaffold, degradable linker, and lipid unit. Several studies [10,11] have reported that synthetic spermine derivatives are effective gene delivery vectors. These polycations bind to DNA electrostatically and form lipoplexes (lipopolyamine and DNA complexes) that are endocytosed by many cell types and deliver DNA with varying degrees of high delivery efficiency and low (acceptable) toxicity [6,12].

Cationic lipids are not only prepared from the conjugation of lipid moieties with linear polyamines. Lehn and co-workers [4] recently reported a novel use of an aminoglycoside scaffold, kanamycin, a polyamine on a cyclic skeleton, incorporated in a lipopolyamine design. Here, we report our work on the design and synthesis of a polyamine gene delivery vehicle based on the aminoglycoside neomycin B (Figure 1a) [13] that can carry up to six amino groups

which are predominantly fully charged (protonated) at physiological pH [14]. Consequently, we considered that this conjugate may condense DNA more efficiently, and may be capable of delivering circular plasmid DNA to hard-to-transfect primary cell lines. Therefore, we have synthesized 3 β -[5''-(aminoethanethiol)-neomycin B] carbamoyl cholesterol (NeoChol) (Figure 1b).

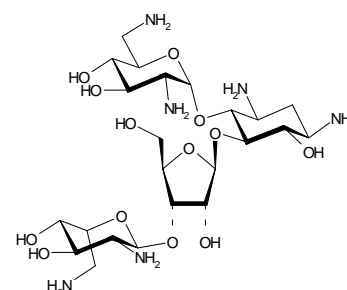


Fig. 1a: Neomycin B

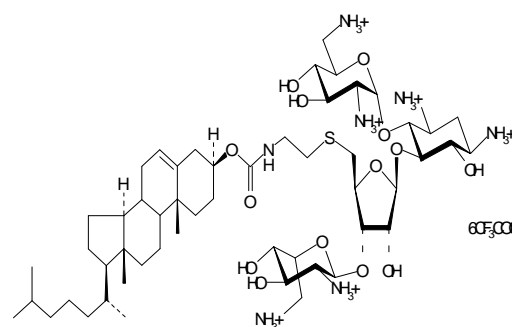


Fig. 1b: 3 β -[5''-(Aminoethanethiol)-neomycin B] carbamoyl cholesterol (hexa-trifluoroacetate salt)

In our preliminary experiments to determine the DNA condensation ability across a series of readily available aminoglycosides (an intercalated ethidium bromide fluorescence quenching assay

compared with a UV $\lambda = 320$ nm light scattering assay), neomycin B displayed the best results. It was the most positively charged of the aminoglycosides we examined. Not entirely unexpectedly, however, free base neomycin B did not deliver pDNA encoding for enhanced green fluorescent protein (EGFP) in (primary) FEK4 skin cells, where its efficiency was only comparable with naked DNA (control, $2 \pm 2\%$). This is possibly due to neomycin B's poor solubility in the organic phase, and such a physicochemical property probably makes it impossible for this hexa-amine to traverse the lipophilic cell membrane. These results indicate that the efficient pDNA binding and condensing abilities displayed by neomycin B are not sufficient to bring about efficient cell transfection. So, six positive charges alone are not enough for transfection, a lipid unit is necessary in the lipopolyamine NVGT vector design.

Our goal is to design and evaluate efficient lipid-aminoglycoside conjugates for the delivery of genes for gene therapy [4,14], and also for the delivery of siRNA molecules to knock down gene expression in mammalian cells [15,16]. We have therefore designed and made a cationic lipid based upon aminoglycoside cationic head-group linked to a lipid moiety e.g. cholesterol or long fatty acid chains. The conjugation of neomycin B via a thioether to cholesterol through a carbamate provides an "intelligent" material which may well overcome the problems still found in poly-nucleic acid delivery [17].

Without any pre-formation of liposomes, NeoChol brings about controlled self-assembly into a scaffold, based on the polycationic moiety, and also facilitates absorptive endocytosis and/or fusion with cell membranes through the lipid regions. NeoChol spontaneously neutralises and condenses negatively charged DNA and RNA, leading to the efficient nanoparticle formation after counterion removal in a thermodynamically favoured step. These bio-nanoparticles can be used for siRNA delivery (using fluorescein-tagged siRNA as the reporter). The self-assembled, non-liposomal complexes were characterised physicochemically by particle size measurement and fluorescence intercalation assay. They were tested, *in vitro*, for transfection efficiency and their cytotoxicity measured in the MTT assay.

MATERIALS AND METHODS

Materials

Chemicals, including, reagents, solvents, buffer, and DNA were routinely purchased from Sigma-Aldrich, UK, except where indicated. Lipofectin and Lipofectamine reagents from Invitrogen (Life Technologies Gibco BRL) and cell cultures materials from Life Technologies (Paisley, Scotland).

Synthesis of NeoChol

For the synthesis of our target 3 β -[5''-(aminoethanethiol)-neomycin B] carbamoyl cholesterol (NeoChol), neomycin B sulfate was purchased from Sigma-Aldrich (U.K.) and, without further purification, was converted into its free base using strongly basic ion-exchange resin. All six primary amine groups on neomycin B were *N*-Boc-protected in a mixed solvent system of DMF-H₂O (6:1, v/v) according to the method of Tor *et al* [15]. The crude product was purified by column chromatographic elution over flash silica gel (DCM-MeOH, 20:1, v/v, 57% yield). Then, in the presence of six secondary alcohol functional groups, the only primary hydroxyl group, on the 5'' position pendant from furanose ring C, was selectively activated using 30 eq. 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) in pyridine at 20 °C for 16 h (monitored by DEPT spectroscopy, the shift of C-5'' at 60.4 ppm moved significantly towards low field at 69.4 ppm on sulfonation). Significantly fewer TPS-Cl eq. were not effective in achieving a practical yield of O^{5''}-(2,4,6-triisopropylbenzenesulfonyl) *N*-hexa-*tert*-butyloxycarbonyl neomycin B. The excess of TPS-Cl was washed out with DCM in a column chromatographic elution over flash silica gel. The desired product showed R_f 0.45 by TLC with mobile phase DCM-MeOH (10:1, v/v). An aminoethanethiol linker to bridge between the neomycin B (polyamine moiety) and the lipophilic moiety (cholesteryl) was introduced. This linker was coupled using (freshly prepared) sodium ethoxide as the base. Up to 45 eq. aminoethanethiol HCl were converted into the free base with NaOEt and used to substitute the

2,4,6-triisopropylbenzenesulfonate moiety. Having introduced the free primary amine, conjugation with cholesteryl chloroformate (1.2 eq.) was carried out in a mixed solvent system of anhydrous THF-DMF (3:1) and triethylamine (1.2 eq.) at 20 °C for 16 h. The product showed R_f 0.63 by TLC (DCM-MeOH, 10:1, v/v). A small sample, dissolved in DCM, was de-protected of all six Boc groups, achieved with TFA (6 eq.) in an ice-saline bath for 2 h with thorough stirring. After several co-evaporations with MeOH, the solid residue was dissolved in water and subsequent lyophilization afforded the desired product, NeoChol (TFA salt), as a fluffy white powder.

Amplification and Purification of Plasmid DNA (pEGFP)

We have chosen to deliver a 4.7-kbp plasmid encoding for enhanced green fluorescent protein (pEGFP), with a molecular weight of about 3.1 MDa (given an average of 330 Da per nucleotide, 660 Da per base pair [18], carrying 9400 negative charges). DNA plasmid encoding enhanced green fluorescent protein (pEGFP) purchased from Clontech was transformed into *Escherichia coli* JM 109 bacterial strain (Promega). The transformed cells were grown in larger quantities of Luria-Bertani (LB) broth supplemented with 125 mg/L ampicillin. pEGFP plasmid was produced in large-scale using HiSpeed plasmid purification Maxi kit (Qiagen) according to the manufacturers protocol. DNA yields and purity were determined spectroscopically ($OD_{260}/OD_{280} = 1.80$ to 1.90 OD, optical density) and by agarose gel (1% agarose) analysis.

DNA Condensation (Ethidium Bromide Fluorescence Quenching Assay)

Each concentration of the DNA stock solutions (approximately 1 μ g/ μ l, 1 ml) was determined spectroscopically (Milton Roy Spectronic 601 spectrometer, 1cm path length, 3 ml cuvette) [18] and 6 μ g (approximately 6 μ l) of DNA was diluted to 3 ml with buffer (20 mM NaCl, 2 mM HEPES, 10 μ M EDTA, pH 7.4) in a glass cuvette stirred with a micro-flea. Immediately prior to analysis, EthBr solution (3 μ l, 0.5mg/ml) was added to the stirring solution and allowed to equilibrate for 10 min. Separately for DeChol aliquots (5 μ l, according to the ammonium/phosphate (+/-) charge ratio required) were then added to the stirring solution and the fluorescence measured after 1 min equilibration using Perkin-Elmer LS 50B luminescent spectrometer ($\lambda_{excit} = 260$ nm and $\lambda_{emiss} = 600$ nm with slit width 5nm) while stirring using an electronic stirrer (Rank Bros. Ltd.) [19, 20]. The total lipopolyamine solution added to the DNA solution did not exceed 5% of the total volume of the solution, so no correction was made for sample dilution. The fluorescence was expressed as the percentage of the maximum fluorescence when EthBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EthBr in solution.

• RiboGreen intercalation assay

RiboGreen solution (Invitrogen, 50 μ l dil. 1 to 20) was added to each well of a 96-well plate containing free siRNA (50 ng) or complexed with NeoChol at different ratios containing in TE buffer (50 μ l, 200mM Tris-HCl, 20 mM EDTA, pH 7.5, in DEPC-treated water) using FLUOstar Optima Microplate Reader (BMG-LABTECH), $\lambda_{excitation}$ 480 nm, $\lambda_{emission}$ 520 nm. The amount of siRNA available to interact with the probe was calculated by subtracting the values of residual fluorescence (RiboGreen without siRNA) from those obtained for each measurement, and expressed as a percentage of the control that contained naked siRNA only, according to the following formula: % free siRNA = $100 \times \text{RiboGreen fluorescence}_{\text{complexes}} / \text{RiboGreen fluorescence}_{\text{naked siRNA}}$.

Gel Electrophoresis

Each sample of plasmid DNA (0.5 μ g), either free or complexed with DeChol at different concentration, was analyzed by gel electrophoresis for about 60 min under 75 V/cm, through an agarose gel (1%) containing EthBr (1 μ g/ml) in Tris- acetate-EDTA 1 \times (40 mM Tris-acetate and 1 mM EDTA) buffer. The (unbound) free DNA in the agarose gel was visualized under UV using GeneGenius (Syngene, Cambridge, UK).

Lipoplex Particle Size

The average particle size for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, was determined using a NanoSight LM10 (NanoSight Ltd, Salisbury, UK). All measurements were carried out on lipoplexes with 1 µg/ml plasmid DNA in HEPES buffer at pH 7.4 in a sample volume 0.2 ml. Results are analysed by the ground-breaking Nanoparticle Tracking Analysis (NTA) software.

ζ-Potential Measurements

The ζ-potential measurements for the lipoplexes formed (at their optimum charge ratio of transfection), after mixing with a vortex mixer, was determined using a Delsa™ Nano Zeta Potential (Beckman Coulter, Buckinghamshire, UK). All measurements were carried out on lipoplexes with 6 µg/ml plasmid DNA in HEPES buffer at pH 7.4 in sample volume 2 ml.

DNase I sensitivity assay

Briefly, in a typical assay, pEGFP plasmid (1 µg) was complexed with the varying amounts of the DeChol using the indicated charge ratios in a total volume of 30 µL in HEPES buffer, pH 7.40, and incubated at room temperature for 30 min on a rotary shaker. Subsequently, the complexes were treated with 10 µL DNase I (at a final concentration of 1 µg/mL) in presence of 20 mM MgCl₂ and incubated for 20 min at 37°C. The reactions were then halted by adding EDTA (to a final concentration of 50 mM) and incubated at 60°C for 10 min in a water bath. The aqueous layer was washed with 50 µL of phenol:chloroform:isoamylalcohol (25:24:1 mixture, v/v) and centrifuged at 10,000 rpm for 5 min. The aqueous supernatants were separated, loaded (15 µL) on a 1% agarose gel (pre-stained with ethidium bromide) and electrophoresed at 100 V for 1 h.

Cell Culture and Transfection Experiments with pEGFP DNA

Two cell lines were used in the transfection experiment, a human primary skin fibroblast cells FEK4 [20], derived from a foreskin explant and a human cervix carcinoma, HeLa derivative and transformed cell line (HtTA). The HtTA cells being stably transfected with a tetracycline-controlled transactivator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV). Cells were cultured in Earle's minimal essential medium (EMEM) supplemented with foetal calf serum (FCS) 15% in the case of FEK4 and 10% in the case of HtTA cells, penicillin and streptomycin (50 IU/ml each), glutamine (2 mM), and sodium bicarbonate (0.2%).

For the transfection (gene delivery) and the resultant gene activity (transfection efficiency), FEK4 and HtTA cells were seeded at 5×10^4 cell/well in 12 well plates in 2 ml EMEM media with FCS for 24 h to reach a plate confluence of 50–60% on the day of transfection, then replaced by 0.4 ml EMEM media with FCS. The complex was prepared by mixing 1 µg of pEGFP with the cationic lipopolyamine in Opti-MEM according to the charge ratio at room temperature for 30 min and then incubated with the cells for 4 h at 37°C in 5% CO₂ in full growth medium (in the presence of serum). Then the cells were washed and cultured for further 44 h in full growth medium at 37°C in 5% CO₂ before the assay.

Levels of enhanced green fluorescent protein (EGFP positive cells) in the transfected cells were detected and corrected for background fluorescence of the control cells using a fluorescence activated cell sorting (FACS) machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

Transfection Experiments in Presence or Absence of Serum

The same as the previous procedures but the complex added to cells in Earle's minimal essential medium (EMEM) (Serum free media) for 4 h at 37°C in 5% CO₂. Then the cells were washed and cultured for further 44 h in full growth medium at 37°C in 5% CO₂ before the assay.

siRNA Transfection experiments

We used two cell lines, a human primary skin fibroblast cell line (FEK4) derived from a foreskin explant cell line and a human cervix carcinoma, HeLa derived and transformed cell line (HtTA). For the transfection, FEK4 and HtTA cells were seeded at 5×10^4 cells/well in 12-well plates in 1 ml EMEM media with FCS for 24 h to reach a plate confluency of 50–60% on the day of transfection. The complex was prepared by mixing siRNA (12.5 pmoles) with the Transfecting agent in Opti-MEM (serum free media, Gibco BRL) according to the optimum concentration, at 20°C for 30 min and then incubated with the cells for 4 h at 37°C in 5% CO₂ using EMEM media with FCS. Then the cells were washed and cultured for a further 44 h in growth medium at 37°C in 5% CO₂ before assaying. Levels of fluorescence in the transfected cells were detected and corrected for background fluorescence of the control cells using a FACS machine (Becton Dickinson FACS Vantage dual Laser Instrument, argon ion laser 488 nm). Data were analyzed with WinMIDI (version 2.8) software. The transfection efficiency was calculated based on the percentage of the cells that expressed EGFP (positive cells) in the total number of cells.

In Vitro Cytotoxicity (MTT) Assay

FEK4 and HtTA cells were seeded in 96 well plates at 8000 cell/well and incubated for 24 h at 37°C in 5% CO₂. Transfecting agent complexed with pEGFP or siRNA was added in the same way as the transfection protocol. After incubation for 44 h, the media was replaced with 90 µL of fresh media and 10 µL of sterile filtered MTT solution (Sigma-Aldrich, UK) (5 mg/ml) to reach a final concentration of 0.5 mg/ml. Then the plates were incubated for a further 4 h at 37°C in an atmosphere of 5% v/v CO₂. After incubation, the media and the unreacted dye were aspirated and the formed blue formazan crystals were dissolved in 200 µL/well of dimethyl sulfoxide (DMSO). The produced color was measured using a plate-reader (VERSAmax) at wavelength 570 nm. The % viability related to control wells containing cells without DNA and/or polymer and is calculated by (test absorbance/control absorbance) × 100 [21]. The same protocol was applied in case of the commercially available reagents Lipofectin and Lipofectamine [22-24].

RESULTS

Synthesis of lipospermines

3β-[5'-(Aminoethanethiol)-neomycin B] carbamoyl cholesterol (NeoChol) (6 x TFA salt) showed LSIMS HR accurate mass data: FAB⁺ m/s found 1086.6727 Da, C₅₃H₉₆N₇O₁₄S requires 1086.6730 [M-6TFA+H]⁺, within 0.3 ppm. It was homogeneous after TFA deprotection of the hexa-Boc synthetic protected intermediate.

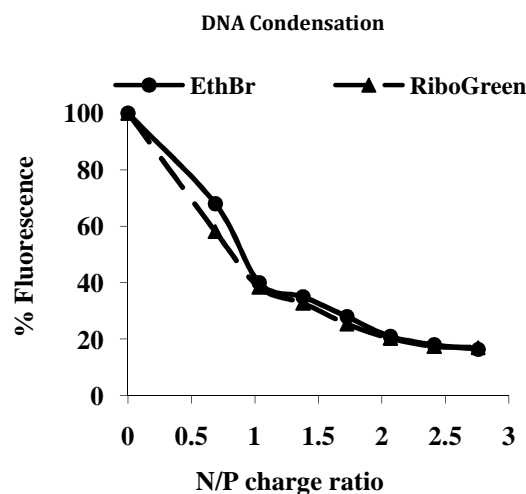


Fig. 2: Plot of EthBr displacement assay of pEGFP and RiboGreen intercalation assay of RNA complexed with NeoChol

The EthBr displacement assay and RiboGreen intercalation assay shows pEGFP DNA and siRNA condensation as a function of increasing the N/P charge ratio (figure 2).

The gel electrophoresis results show that all spermine conjugates were able to condense pEGFP DNA efficiently (as a result of neutralization of DNA phosphate negative charges by the lipopolyamines ammonium positive charges) at their optimised respective charge ratios (n/p) of transfection by completely inhibited the electrophoresis mobility of plasmid DNA.

Lipoplex Particle Size and Zeta-Potential Measurements

The particle size and ζ -potential characterization measurements were carried out on the lipoplexes at their optimum N/P charge ratio of transfection. Particle size characterization by laser diffraction showed that the average particle size of the surface charge, as determined by ζ -potential measurements. ζ -Potential is an important parameter helping to predict the stability of the formulation as well as the ability of the positively charged particles to interact with cell membranes. ζ -Potential depends on several factors, including pH, ionic charge, ion size, and concentration of ions in solution. The formed nanoparticles are considered to be stable when they have pronounced ζ -potential values, either positive or negative, but the tendency to aggregate is higher when the ζ -potential is close to zero the measured zeta potential for the naked DNA is -1.02 and +18.05 for condensed DNA at optimum N/P ratio.

For the nanoparticles, the average particle size was 200 nm for pDNA and 120 nm for siRNA Nase I Protection

The DNase I protection experiment carried using plasmid DNA complexed with the different N/P ratios lipopolyamines. Reasonably intense undigested DNA bands were detected in the gel as a control and no band for uncondensed DNA which digested by DNase I as negative control. We performed the DNase protection experiment using different N/P ratios of DeChol which mean that DNA condensed with these lipopolyamines completely protected from DNase I enzyme (figure 3).

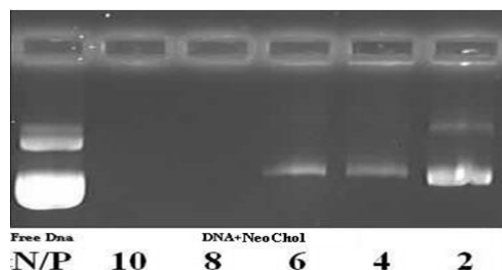


Fig. 3: Gel electrophoresis assay of pEGFP complexed with NeoChol at different N/P ratios

Transfection Experiments and *In Vitro* Cytotoxicity

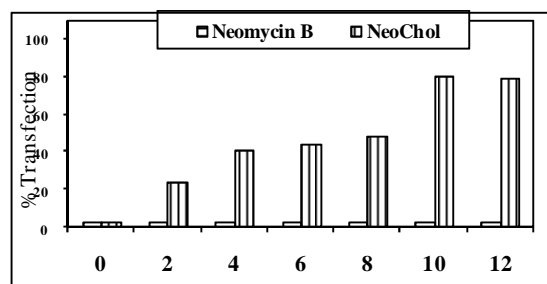


Fig. 4: Lipofection of FEK4 cell lines transfected with DNA complexed with neomycin B and NeoChol at different N/P charge ratios

Fig. 4: shows that neomycin B did not transfect the FEK4 primary cell line (although it did condense pDNA). However, NeoChol, as a function of increasing N/P charge ratio showed efficient DNA transfection was achieved at N/P 10 and 12.

Fig. 4: show efficient transfection efficiency of NeoChol even in presence of serum. Figures 5 and 6 show efficient NeoChol transfection with good viability comparable to the commercial transfecting agent LipoGen for DNA delivery and TransIT for siRNA delivery

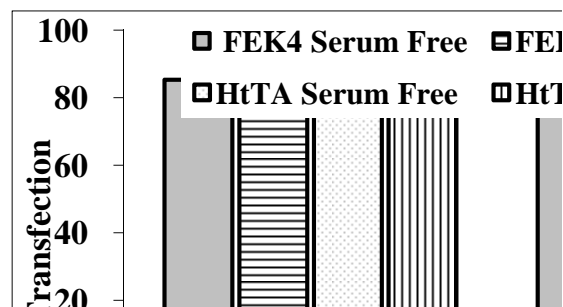


Fig. 5: Lipofection of FEK4 and HtTA cell lines transfected with DNA complexed with NeoChol in presence and absence of serum.

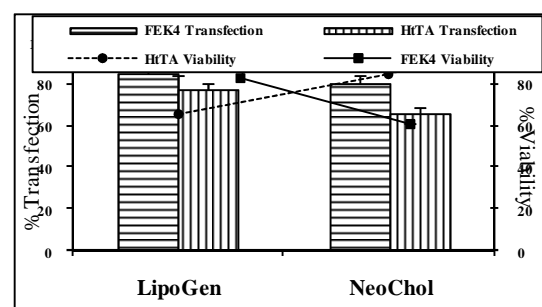


Fig. 6: Lipofection and viability of FEK4 and HtTA cell lines transfected with DNA complexed with NeoChol

The gene delivery efficiency and cell viability of NeoChol were examined with an FEK4 cell line (primary skin fibroblast, derived from a foreskin explant). As positive controls for these experiments, we compared the delivery efficiency and toxicity of our conjugate with N^4, N^9 -dioleoyl spermine (Lipogen) that is an established gene delivery agent.¹⁰ Untransfected cells, "naked" pDNA, and the parent drug, neomycin B, were used as the negative controls in these experiments. The transfection efficiency was calculated based on the percentage of cells that expressed EGFP (positive cells) in the total number of cells (Figure 4). Under the same conditions when neomycin B did not show any (2 ± 2 %) cell transfection ability, using the same FEK4 primary skin cell line, NeoChol showed 82 ± 3 % transfection ($n = 9$) at ammonium/phosphate (N/P) charge ratio 10 (Figure 4). An acceptable level of cell toxicity was established in an MTT assay, showing 58.2 ± 3.8 % cells survival. The results from this transfection assay indicate that a combined consequence of the good pDNA binding and condensing affinity displayed by the neomycin B moiety and the lipophilic property of the cholesteryl unit is efficient primary cell line transfection, when compared to the parent molecule, neomycin B or to naked DNA (Figure 4). Furthermore, NeoChol (at N/P charge ratios 10-12) is at least as efficient as commercially available Lipogen (Figure 6).

siRNA Transfection

Figure 7 shows efficient siRNA delivery and cell viability as good as the commercial compound Trans IT for both primary FEK4 and Cancer HtTA cell lines

In summary, novel therapeutic poly-nucleic acids may yield revolutionary advances in modern medicine, provided that a safe and effective delivery method is discovered. Here, we report the synthesis and biological activity of NeoChol that exhibits high gene delivery efficiency with low toxicity in a cancer cell line and primary skin cell line as good as the commercially available Lipogen, and comparable with TransIT-TKO® for siRNA delivery.

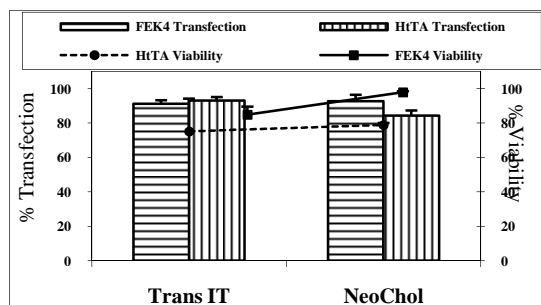


Fig. 7: Delivery and viability of FEK4 and HtTA cell lines transfected with fluorescent siRNA complexed with NeoChol

CONCLUSIONS

We have demonstrated that a synthetic cationic lipid, a conjugate derived from neomycin B antibiotic and cholesterol is highly efficient for the in vitro delivery of DNA (65-80%) and fluorescent siRNA (85-90%). Good cell viability was also observed compared with TransIT (for RNA) (80-95%) and LipoGen (for DNA) (60-85%) delivery. NeoChol is a candidate medicine worthy of further development due to its efficient gene and siRNA delivery with low toxicity. It is a novel, efficient non-viral vector in the presence of serum.

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